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EFFECTS OF HIRUDIN ON HORMONE-INDUCED ACTIVATION OF NONENZYMATIC FIBRINOLYSIS DURING IMMOBILIZATION STRESS

F. B. Shapiro, I. P. Baskova, L. U. Cherkesova, L. A. Lyapina and M. D. Gol'dovskaya

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EFFECTS OF HIRUDIN ON HORMONE-INDUCED ACTIVATION OF NONENZYMATIC FIBRINOLYSIS DURING IMMOBILIZATION STRESS

F. B. Shapiro, I. P. Baskova, L. U. Cherkesova, L. A. Lyapina and M. D. Gol'dovskaya
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In recent years in a number of works done under the direction of B. A. Kudr- /1567*
yashov it has been shown, that there is hormonal conditioning for the activation of the function of the anticoagulation system under conditions of stress. Evidence of this is the fact, that an increase in the nonenzymatic fibrinolytic activity (NEFA) of the chief index that characterizes stimulation of the anticoagulation system [4] during stress is regulated by the hormonal status of the organism, a condition which has changed under these circumstances and is determined by the emission of adrenalin, adrenocorticotropic hormone and glucocorticoids [7, 8]. If the mechanism of the effect of adrenalin on NEFA may be considered as established (adrenalin stimulates thrombinogenesis and by the very fact excites the anticoagulation system accompanied by an intensification of NEF), the mechanism of the effect of the ACTH is still unclear, although there are some data to indicate that it stimulates the emission of heparin into the blood [9].

In the present work we have set ourselves the task of studying the difference between the mechanism of the effect of the ACTH and adrenalin under conditions which block the formation of thrombin in the organism. For this purpose we used a purified preparation of hirudin, a specific inhibitor of thrombin, which has the capability /1568
of almost instantly combining with thrombin to form an inactive complex. In this context one should undoubtedly find especially interesting the data on the effect of ACTH against a background of hirudin introduced into the organism, to the extent that they would provide new material for characterizing its stimulating activity on nonspecific fibrinolytic activity in a stress situation. In fact, one might foresee the effect of the simultaneous administration of adrenalin and hirudin to a certain degree.

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* Numbers in the margin indicate pagination in the foreign text.

TABLE I. AVERAGE AND NONENZYMATIC FIBRINOLYTIC ACTIVITY OF BLOOD PLASMA WITH 30 MINUTES IMMOBILIZATION (MM^2)

Group of animals	Number of animals	Average fibrinolytic activity (A)	Nonenzymatic fibrinolytic activity (B)		B×100/A	
			sigma-aminocaproic acid	SBTI	sigma-aminocaproic acid	SBTI
With immobilization	7	75.4±10.2	23.3±1.5	22.9±1.7	32.2±3.0	32.6±3.9
Without immobilization	8	102.1±11.4	48.5±3.4	49.2±3.2	50.9±5.1	52.0±6.3

TABLE II. AVERAGE AND NONENZYMATIC FIBRINOLYTIC ACTIVITY OF BLOOD PLASMA WITH ADMINISTRATION OF HIRUDIN TO IMMOBILIZED ANIMALS (MM^2)

Group of animals	Number of animals	Average fibrinolytic activity (A)	Nonenzymatic fibrinolytic activity (B)	B×100/A
Without immobilization	14	52.4±12.2	21.0±1.6	39.6±4.3
Immobilization + saline solution 30 min before blood sampling	13	99.4±11.3	60.8±2.9	65.7±3.9
Immob.+hirudin 30 min before blood sampling	12	141.0±16.2	68.9±11.1	45.6±5.2
Immobilization + saline solution 5 min before blood sampling	5	76.0±5.7	44.6±3.5	59.8±5.3
Immobilization + hirudin 5 min before blood sampling	5	139.4±6.8	47.0±2.9	33.5±2.1

Method

The work was done on mongrel male rats weighing 200-250 g. The stress situation was created by 30 minutes of immobilization (they were fastened to a table). Blood sampling was done from the jugular vein with sodium nitrate in the ratio of 9:1. We used an ACTH-active preparation obtained from the hypophyses of swine (activity 60 units/mg), and hirudin, purified preparation, obtained from the heads of medicinal leeches, using a method we had developed [1]. The activity of the preparation used in the experiment was 1500 antithrombin units/mg. The ACTH was administered to the rats intraperitoneally at a dosage of 5 units/200 g. The hirudin was administered

TABLE III. FIBRINOLYTIC ACTIVITY OF BLOOD WITH ADMINISTRATION OF HIRUDIN DETERMINED BY THE BIDWELL METHOD

Group of animals	Number of animals	Fibrinogen (mg%)	Fibrinolytic activity (%)
Without immobilization	23	344.3 ± 25.6	9.4 ± 2.6
With immobilization	22	316.4 ± 17.1	21.7 ± 3.5
Immobilization + saline solution 30 min before blood sampling	8	224.4 ± 46.7	28.0 ± 3.5
Immobilization + hirudin 30 min before blood sampling	8	150.0 ± 37.0	52.5 ± 8.6

TABLE IV. FIBRINOLYTIC ACTIVITY OF BLOOD WITH HIRUDIN INCUBATION (MM^2)

Experimental conditions	Number of tests	Average fibrinolytic activity (A)	Nonenzymatic fibrinolytic activity (B)	$\frac{Bx100}{A}$
Incubation 1 ml whole blood with 0.3 ml saline solution	4	68.5 ± 9.0	50.7 ± 12.3	70.7 ± 10.8
Incubation 1 ml whole blood with 0.3 ml hirudin (activity 37 units)	4	64.0 ± 9.9	50.0 ± 8.5	75.5 ± 8.2

TABLE V. AVERAGE AND NONENZYMATIC FIBRINOLYTIC ACTIVITY OF BLOOD PLASMA 10 MINUTES AFTER ADMINISTRATION OF HIRUDIN-THROMBIN COMPLEX (MM^2)

Group of animals	Number of animals	Average fibrinolytic activity (A)	Nonenzymatic fibrinolytic activity (B)	$\frac{Bx100}{A}$
Saline solution	10	68.1 ± 4.3	27.3 ± 3.5	36.8 ± 3.0
Thrombin	5	138.2 ± 14.7	85.6 ± 10.8	62.0 ± 5.0
Hirudin-thrombin	6	149.2 ± 17.6	53.7 ± 7.3	36.0 ± 2.1

intravenously at a dosage of 55 units/200 g. Adrenalin was administered IV at a dosage of 5 micrograms/200 g. Total volume of the administered substances was 1 ml; in all cases a correspondingly equal amount of saline solution was administered IP or IV to the control. Enzymatic fibrinolytic activity was determined using Bidwell's method [12]. For differential determination of average fibrinolytic activity and NEFA we used platelets of nonstabilized fibrin according to the method of B. A. Kudryashov, L. A. Lyapina and I. P. Baskova [6]. As plasmin inhibitors we

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TABLE VI. AVERAGE AND NONENZYMATIC FIBRINOLYTIC ACTIVITY OF BLOOD PLASMA WITH ADMINISTRATION OF ADRENALIN AND HIRUDIN TO IMMOBILIZED ANIMALS (MM^2)

Group of animals	Number of animals	Average fibrinolytic activity (A)	Nonenzymatic fibrinolytic activity (B)	$\frac{Bx100}{A}$
Immobilization + saline solution	6	87.3±2.7	42.5±4.0	48.1±3.4
Immobilization + adrenalin	8	232.7±19.2	140.6±8.1	61.7±2.5
Immobilization + adrenalin + hirudin	8	167.4±18.7	71.1±8.0	41.6±1.9

TABLE VII. AVERAGE AND NONENZYMATIC FIBRINOLYTIC ACTIVITY OF BLOOD PLASMA WITH ADMINISTRATION OF ADRENOCORTICOSTEROID HORMONE AND HIRUDIN TO IMMOBILE ANIMALS (MM^2)

Group of animals	Number of animals	Average fibrinolytic activity (A)	Nonenzymatic fibrinolytic activity (B)	$\frac{Bx100}{A}$
Immobilization + saline solution	7	83.1±3.5	39.0±3.3	46.9±3.2
Immobilization + adrenocorticosteroid hormone	10	160.2±10.2	102.6±5.6	64.9±3.1
Immobilization + adrenocorticosteroid hormone + hirudin	16	187.5±15.6	131.0±11.1	68.0±0.3

used sigma-aminocaproic acid (put out by "Chemafol") in the form of a 6% solution and a trypsin inhibitor from soy beans SBTI (put out by "Reanal") with an activity of 10,000 antitryptin units/mg. The SBTI was dissolved in a saline solution in the amount of 4 mg in 1 ml.

Results and Evaluation

Testing the Possibility for the Use of SBTI to Inhibit Plasmin Activity When Determining Nonenzymatic Fibrinolytic Activity of Blood Plasma

As we know, when NEFA is determined by the fibrin platelet method [6], the plasmin inhibitor used is a 6% sigma-aminocaproic acid. The extensive use of this method has shown, that the accuracy of the results obtained depends to a great degree upon the quality of the preparations of sigma-aminocaproic acid, which are often

fairly unstable. Therefore it was undoubtedly important to test the possibility of using another plasmin inhibitor, SBTI, instead of sigma-aninocaproic acid. For this reason we conducted a special experiment which produced the previously described [5, 7, 8] effect of an increase in NEF during immobilization stress, where both sigma-aminocaproic acid and SBTI were used as inhibitors of plasmin activity. The results show (Table I), that SBTI in concentrations of 4 mg/1 ml of saline solution blocks EFA to the same extent as a 6% solution of sigma-aminocaproic acid. This made it possible for us in all further work to use SBTI for determining NEFA.

The data shown in Table I indicate, that under the conditions of our experiment the animals subjected to 30 minutes of immobilization present a reliable increase in the average fibrinolytic activity of blood plasma, but in this situation there is a regularly greater increase in NEFA. Thus, if average fibrinolytic activity in these animals increases 1.4 times, NEFA increases more than 2 times, so that the proportion of NEF in average fibrinolytic activity during stress increases 1.5 times.

Fibrinolytic Activity When Hirudin Is Administered during Stress /1569

Inasmuch as we used hirudin in solving our problem, it must first of all be made clear, whether it in itself produces any changes in the fibrinolytic activity of the blood. For this purpose animals subjected to immobilization received hirudin the instant they were immobilized (30 minutes before blood sampling), as well as at the end of immobilization (5 minutes before blood sampling).

As the results of the experiment show (Table II), the administration of hirudin induces a change in fibrinolytic activity and the effect is the same whether it is given at the beginning or at the end of the stress period. When hirudin is administered there is a 1.5 fold increase in the average fibrinolytic activity as compared with the average fibrinolytic activity of the controls that received only the saline solution. In respect to NEFA there is no difference from the controls, if we consider absolute quantity. The amount of NEFA in the average fibrinolytic activity following administration of hirudin, of course, drops significantly and is practically the same as the proportion of NEFA in the average fibrinolytic activity for rats not subjected to the stress factor.

An increase in fibrinolytic activity when hirudin was administered was also noted when the Bidwell method of determination was used. As may be seen from the data in Table III, fibrinolytic activity in animals given hirudin was 1.9 times greater than in the controls ($0.05 > p > 0.01$). /1570

An increase in average fibrinolytic activity takes place only when hirudin is administered in vivo. The incubation of whole blood with hirudin in vitro has shown, that in such a case there is no increase in the average fibrinolytic activity. As is seen from the data in Table IV, the average fibrinolytic activity and NEFA of blood samples incubated with hirudin and with saline solution is practically the same; in the two cases there is no difference in the proportion of NEFA in the average fibrinolytic activity.

We know, that hirudin combines with thrombin to form in the organism a nondissociating equimolar complex devoid of coagulating and esterase activity [13]. It might be supposed, that the effect we obtained, where active fibrinolytic activity rose with the administration of hirudin to animals in a stress situation, was conditioned by the action of this complex formed through an interaction of hirudin with endogenous thrombin.

If the hirudin-thrombin complex possessed the capability of increasing average fibrinolytic activity, possibly its effect would have also appeared when the previously formed complex was administered to the organism. We tested this hypothesis by administering to the animals a complex formed by neutralizing of 55 units of thrombin and 55 units of hirudin (the hirudin dosage used in all the experiments). One group of controls received a saline solution, the other 55 units of native thrombin. As may be seen from the data in Table V, when the hirudin-thrombin complex was administered the proportion of NEFA in the average fibrinolytic activity remained the same as when the control received the saline solution. However, IV administration of thrombin is accompanied by a significant increase in this proportion and this is a feature, as we know, characterizing the activity of the anticoagulation system [4].

Thus, these data are evidence, that the hirudin-thrombin complex did not induce an increase in NEFA characteristic of excitation of the anticoagulation system. /1571 It is probable that when the complex was administered, the observed matching increase in average fibrinoltyic activity and NEFA could be explained by a nonspecific lytic

action on the part of the complex affecting nonstabilized fibrin platelets. This is indicated by the following fact: The hirudin-thrombin complex is incapable of coagulation activity in reference to a fibrinogen and completely devoid of benzoylarginin-methyletheresterase acitivity. At the same time, when the complex affects a fibrin platelet, we note a rather significant lysis zone, following 2 hours of incubation at 37° of 54 mm^2. However, when the platelets are affected by the components making up the complex, there is no lysis.

Fibrinolytic Activity When Adrenalin and Hirudin are Given during Stress

As has already been shown, the mechanism of the action of adrenalin on the activation of the function of the anticoagulation system may be considered as established. Adrenalin affects various aspects of the blood coagulation process by stimulating thrombinogenesis and accelerating coagulation [3, 14]. By the same token it induces a reflex excitation of the anticoagulation system and consequently an increase in NEFA [8]. With this as our starting point we would expect that, inasmuch as hirudin blocks the formation of thrombin, the simultaneous administration of adrenalin and hirudin would not induce an increase in NEFA. To test this we gave animals subjected to immobilization both hirudin and adrenalin; one control group received adrenalin and the other a saline solution. As we see from the data in Table VI, the administration of adrenalin produced an increase in the absolute quantity of NEFA by 3.6 times, and its proportion in the average fibinolytic activity by 1.3 times in comparison with the controls that had received a saline solution. In respect to the administration of adrenalin against a background of hirudin, we see that, at the dosages we used, the effect of adrenalin was considerably attenuated by the hirudin although not completely eliminated. /1572

This is borne out by the following facts: in animals that had received adrenalin and hirudin the level both of average fibrinolytic activity and NEFA was from 1.8-2 times higher than in the controls that had received a saline solution. However the NEFA in this case was reliably lower ($p < 0.001$) than for administration of adrenalin alone and, a very important fact, there was the absence of something characteristic of stimulation of the anticoagulation system, i. e. an increase in NEFA, and its proportion in the average fibinolytic activity did not differ from that in the controls that had received a saline solution.

Fibrinolytic Activity with ACTH and Hirudin in a Stress Situation

We know, that in the case of immobilized animals, that receive ACTH and by the very fact experience intensified stress, the absolute quantity of NEFA and its proportion in the average fibrinolytic activity increases to an extent significantly greater than in animals subjected to immobilization alone [7, 8]. If the ACTH stimulates the anticoagulation system through the activation of thrombinogenesis, one would expect, that against a background of hirudin, which blocks thrombin, the action of the ACTH would not appear and the NEFA in the animals that had received the ACTH and hirudin would be the same as in the controls that had not received the ACTH. To test this we administered an ACTH and hirudin simultaneously to animals subjected to immobilization, while the controls received separately ACTH and saline solution. The results are presented in Table VII.

As we may see from these data, the immobilized animals that had been given ACTH and hirudin presented average fibrinolytic activity and NEFA (both the absolute amount and the proportion in the average fibrinolytic activity) which increased in practically the same measure as did that of the controls that had received only the ACTH. In comparison with the immobilized animals that had received the saline solution, the animals which had received ACTH (with or without hirudin) presented an /1573
absolute quantity for NEFA that increased by 2-2.8 times and its proportion in the average fibrinolytic activity increased by 1.4-1.5 times.

Putting together the entire presentation, we may draw the following conclusion: the use of the hirudin preparation, a specific thrombin inhibitor, indicates that, when it is simultaneously administered with ACTH to animals subjected to immobilization stress, there is observed the same increase in NEFA that takes place when only the ACTH is administered, i. e. the hirudin does not remove and does not diminish the stimulating activity of the ACTH as it affects NEFA. This is evidence of the fact, that the ACTH has an effect on NEFA in particular not through thrombinogenesis and this is in accord with the previously described fact of heparin emission into the blood due to the action of the ACTH [9]. Undoubtedly one must not suppose, that the mechanism of the activity of the ACTH in respect to the activity of the anticoagulation system is thus fully explained, since this problem requires further research, particularly in connection with the inconsistency of the literature data on the effect of the ACTH on the process of blood coagulation [2, 10, 11].

In respect to the effect of adrenalin the results of our experiments completely agree with the opinion of most researchers about the fact, that it stimulates thrombinogenesis [3, 14] and by the very fact excites the anticoagulation system. In administering hirudin and adrenalin together, despite our somewhat fortuitous selection of dosages, we obtained a reliable reduction in the amount of NEFA as compared with NEFA associated with the administration of adrenalin alone, even though the effect of the stimulating action of adrenalin was not completely eliminated. It might be suggested, that some other pattern of dosages of adrenalin and hirudin would result in complete blockage of the stimulating effect of exogenous adrenalin. However a different thought naturally suggests itself: inasmuch as the administration of the hirudin-thrombin complex, as we have seen, induces a rise in the level of average fibrinolytic activity and NEFA (Table V), possibly in an experiment with the simultaneous administration of adrenalin and hirudin the results would be precisely the effect of the endogenous hirudin-thrombin complex that had been formed.

In respect to the effect of hirudin itself, we have established that, when it is administered to animals subjected to immobilization stress, it induces an increase in enzymatic fibrinolytic activity, while the absolute figure for NEFA remains the same as in the animals that received a saline solution. The proportion of NEFA in the average fibrinolytic activity, of course, is reduced and reaches a level characteristic of animals not subjected to stress. Variations in the results obtained with IV administration of hirudin and the hirudin-thrombin complex may be the result of a quantitative difference. It is obvious, that the amount of endogenous thrombin formed under the conditions of our experiment was inadequate for the amount of hirudin in our doses and therefore the amount of hirudin-thrombin complex formed in the organism was immeasurably less than the amount of the complex which we introduced exogenously. Therefore one may conclude, that in the experiment with the administration of hirudin we produced the effect of the excess hirudin that remained free.

At the present time it is difficult to say, what explanation should be given for the capability of hirudin to induce an increase of enzymatic fibrinolytic activity. One may simply suggest, that hirudin activates some proteolytic enzymatic systems that have a lytic effect. In the end the fact that the administration of hirudin did not induce changes in NEFA may, in particular, be interpreted in the /1574
following way: an increase in NEFA due to the effect of an ACTH in stressed animals, which, as has been shown, is not reduced by hirudin, compensated for a reduc-

tion in NEFA RESULTING FROM hirudin removal of the stimulating effect of adrenalin on NEFA.

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